Complete correction of the enzymatic defect of type I Gaucher disease fibroblasts by retroviral-mediated gene transfer

(gene therapy/vector/expression)

JOSEPH SORGE, WANDA KUHLE, CAROL WEST, AND ERNEST BEUTLER

Scripps Clinic and Research Foundation, Department of Basic and Clinical Research, 10666 North Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT Glucocerebrosidase cDNA and the neomycin-resistance gene (*neo*) were cloned into a retrovirus vector. Mouse fibroblasts infected with this vector expressed human glucocerebrosidase, which was readily distinguished from the mouse enzyme using mouse monoclonal anti-glucocerebrosidase antibodies. Cultured fibroblasts and transformed lymphoblasts from patients with type I Gaucher disease were infected with the retrovirus rescued from the mouse fibroblasts by a helper virus. Transformed cells were selected with the antibiotic G418. The enzyme activity of cells infected with virus containing glucocerebrosidase cDNA was restored to normal, while uninfected cells or cells infected with virus containing only the *neo* gene did not produce glucocerebrosidase.

Gaucher disease is a glycolipid storage disorder caused by an inherited deficiency of the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45) (1, 2). Most attempts at treating Gaucher disease have been disappointing. Enzyme replacement therapy, thought initially to benefit patients with Gaucher disease (3), has shown few objective signs of efficacy (4-7). Although transplantation of allogeneic bone marrow has the potential of curing the disease (8, 9), it is associated with high morbidity and mortality. Spleen (10) and kidney (11) transplantation have been unsuccessful treatments.

Advances in molecular biology open a new avenue for the treatment of this disorder: gene transfer. Gaucher disease is an important candidate for human gene transfer because the phenotypic cellular defect is manifested only in the cells of the macrophage lineage: the common "adult" form of the disease does not affect the central nervous system. Attempts at allogeneic bone marrow transplantation suggest that restoration of normal glucocerebrosidase activity in bone marrow stem cells represents effective treatment of the disease (9). Human glucocerebrosidase cDNA has been cloned (12, 13) and by introducing this cDNA in an expressible form into fibroblasts and lymphoblasts from a patient with Gaucher disease, we have demonstrated the formation of normal levels of glucocerebrosidase. A preliminary report of this work has been published (14).

MATERIALS AND METHODS

Monoclonal Antibodies. The production and characteristics of the mouse monoclonal antibodies used in these investigations are described in detail elsewhere (15).

Cultured Fibroblasts. NIH 3T3 mouse fibroblasts obtained from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium supplemented with 5% (vol/vol) calf serum. Cultured skin fibroblasts (designated HW) were obtained from a patient with typical type I Gaucher disease.

Insertion of the Full-Length Glucocerebrosidase cDNA into the Retrovirus Vector. The production of a full-length human glucocerebrosidase cDNA clone (12, 16) and the construction of the amphotropic retrovirus vector, cistor (17), have been described. We inserted a 3'-splice site, obtained from a 441-base-pair *Xba* I fragment from the envelope gene of Moloney murine leukemia virus (18, 19) between the long terminal repeats (LTRs). The neomycin resistance gene from Tn5 was then inserted downstream of the 3'-splice site (Fig. 1). The full-length glucocerebrosidase cDNA clone was inserted into a unique *Xba* I site between the 5'- and 3'-splice sites. RNA expression of *neo* and glucocerebrosidase genes is controlled by the LTR. RNA splicing presumably allows the translation of both proteins from the same RNA precursor.

Transfection of Mouse Fibroblasts and Infection of Human Gaucher Disease Fibroblast. NIH 3T3 cells that provide amphotropic helper functions (17) were transfected with 15 μ g of the glucocerebrosidase retrovirus vector DNA following the methods of Wigler *et al.* (20). After 48 hr the cells were placed in medium containing the antibiotic G418 at 350 μ g/ml (21). Only cells expressing the neomycin resistance gene survived this selection. The cells were amplified, and the culture medium was used as a source of virus containing the GC gene. Filtered culture medium from virus-producing cells was then added to cultures of human Gaucher disease fibroblasts in the presence of polybrene (8 μ g/ml). After 48 hr, the cells were placed under G418 selection (350 μ g/ml). Resistant cells were pooled and grown to confluency. Gaucher disease fibroblasts were also infected with a retrovirus vector containing the neomycin resistance gene but lacking the GC gene as a control.

Measurement of Glucocerebrosidase Activity and Antigen. Fibroblasts were harvested after reaching confluency by washing the dishes with phosphate-buffered saline (PBS; 10 mM potassium phosphate, pH 7.4/135 mM NaCl) and scraping the cells into 5-10 ml of PBS with a rubber policeman. After pelleting by centrifugation, the cells were resuspended to $\approx$ 25% (vol/vol) in PBS and sonicated for 10 sec at 25 W. Triton X-100 was added to a concentration of 1%, and sodium taurodeoxycholate was added to 0.5%; and, after 10 min on ice, the cells were centrifuged at $25,000 \times g$ for 10 min.

Glucocerebrosidase activity was measured as β -glucosidase as described by Raghavan *et al.* (22), and β -galactosidase and β -N-acetylglucosaminidase were measured as described (23). Enzymatic activity remaining in solution after immunoprecipitation with antibody specific for human β -glucocerebrosidase was carried out as follows: 10 μ l of the $25,000 \times g$ supernatant was incubated overnight at 4°C in a 50- μ l system containing 1% bovine serum albumin and 15-30 μ g of monoclonal antibodies to human β -glucosidase or MOPC-21 ascites fluid as control. Fifty microliters of 10% (wt/vol) protein A (IgG-sorb, The Enzyme Center, Malden, MA) was added, and the tubes were rotated for 30 min at 22°C. Enzymatic activity was determined in supernatants after spinning at $12,000 \times g$ in an Eppendorf centrifuge for 3

FIG. 1. The viral construct used in these studies. GC cDNA, the full coding region of the glucocerebrosidase cDNA. The 5'- and 3'-RNA splice sites are shown.

min. Proteins were determined by the method of Lowry *et al.* (24).

Southern Blotting. Procedures for electrophoresis, blotting, and hybridization were carried out as described (25).

RESULTS

Murine NIH 3T3 fibroblasts expressing amphotropic virus helper functions were transfected with plasmids containing the upstream and downstream LTRs, the *neo* gene, and the glucocerebrosidase cDNA. The acid β -glucosidase activity of the G418-resistant fibroblast extracts was measured before and after treatment with a monoclonal anti-human glucocerebrosidase antibody. As shown in Fig. 2, human enzyme activity was present in mouse fibroblasts that had been transfected with the glucocerebrosidase cDNA and absent from control mouse fibroblasts. The enzyme activities of untreated Gaucher disease fibroblasts, of Gaucher disease fibroblasts infected with a virus containing the *neo* but not the GC gene, and of fibroblasts containing both the *neo* and glucocerebrosidase genes are shown in Fig. 3. The enzyme activity of the Gaucher disease fibroblasts after treatment with the virus containing the glucocerebrosidase cDNA was essentially that of normal cells. The same result was found when lymphoblasts from type I Gaucher patient were infected with the same virus preparation (Fig. 3).

FIG. 2. The effect of transfection of the *neo* and glucocerebrosidase genes into mouse fibroblasts. The bars represent the amount of human enzyme detected—i.e., the decrease in enzyme activity observed when the cell extract was treated with mouse monoclonal anti-human glucocerebrosidase antibody. In the case of the control cells, values $\pm$ 1 SD of three replicate samples are shown. —, Cells infected with control retrovirus. +, Cells infected with glucocerebrosidase cDNA containing retrovirus. β -Gal, β -galactosidase. β -Hex, β -hexokinase. ND, not determined.

Fig. 4 shows a Southern blot of human DNA probed with a human glucocerebrosidase cDNA. *EcoRV* digestion releases a 5.1-kilobase (kb) fragment from the retroviral vector and a 17.5-kb fragment from human genomic DNA. DNA from the Gaucher disease fibroblasts whose enzyme activity had been restored to normal is shown in lane C. The 5.1-kb band representing the viral glucocerebrosidase cDNA sequence is present as well as the normal genomic 17.5-kb band. In contrast, cultured fibroblasts from the same Gaucher disease patient infected with virus that had undergone repeated passage showed only the genomic band (lane B). Using a *neo* probe (data not shown) *neo* sequences could be found in smaller DNA fragments, indicating that the GC gene but not the *neo* gene had been deleted from the virus. By Southern blotting using the *neo* gene as a probe, there were only two classes of deletion mutants found in numerous cultures. We sequenced one of these deletion mutants and found the 5' end of the deletion to be nucleotide-273 of the glucocerebrosidase cDNA (12). The 3' end of the deletion was nucleotide-2116 of the glucocerebrosidase cDNA (12). The 3' end of the deletion is within the 3'-nontranslated region. We have not observed proviral DNA rearrangements or deletions after cell passage, which is consistent with retroviral provirus inheritance.

DISCUSSION

Insertion of the human glucocerebrosidase cDNA into mouse fibroblasts resulted in the appearance of human enzyme activity. The human enzyme was removed by a mouse monoclonal antibody that did not react with mouse glucocerebrosidase protein. When glucocerebrosidase-encoded viral mRNA from the mouse cells was packaged into retroviral particles, it was possible to insert the gene into fibroblasts and lymphoblasts from patients with Gaucher disease. The cells were subjected to selective pressure with G418, and the surviving fibroblasts and lymphoblasts were found to have normal amounts of glucocerebrosidase activ-

FIG. 3. The reconstitution of glucocerebrosidase activity in cultured Gaucher disease fibroblasts from patient H and lymphoblasts from patient F. For normal human fibroblasts and lymphoblasts (open bars) values ± 1 SD of three replicate measurements are shown. Gaucher disease fibroblasts and lymphoblasts are shown as cross-hatched bars. When more than one assay was performed, the mean values of individual assays are shown as closed circles. U, Uninfected cells; V, virus-infected cells; -GC, virus containing only the *neo* gene; +GC, virus containing both *neo* and glucocerebrosidase gene; β -Gal, β -galactosidase; β -Hex, β -hexokinase.

ity. The fact that the proviral structure and enzymatic activity were stable after many cellular passages is encouraging, since any gene delivery system to be used in gene therapy would have to be stable. From previous experience, limiting viral replication to one or two cycles minimizes formation of a high percentage of deleted virus genomes. However, a system that permits multiple viral replication cycles runs the risk of

generating mutants that have lost the gene of interest. Such mutants were encountered when virus subjected to multiple passages in mouse cells was used to infect human fibroblasts.

Type I Gaucher disease is an unusually good candidate for gene replacement therapy. Since we have found glucocerebrosidase antigen in all Gaucher fibroblast lines that we have studied (15), an untoward immunologic reaction to cells producing the normal human enzyme would not be likely in these patients. In type I disease, which is by far most common, neurologic damage does not occur, and the potential for salvage seems unusually high. Yet in its more severe forms type I disease may produce early death, and the assumption of a significant risk by the patient who is severely afflicted seems justified.

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FIG. 4. Southern blot using glucocerebrosidase (GC) cDNA probe. Lane A, 10 μ g of GC-*neo* viral plasmid digested with *EcoRV*; lane B, 10 μ g of DNA digested with *EcoRV* from type I Gaucher cells infected with glucocerebrosidase retrovirus after many viral passages; lane C, 10 μ g of DNA digested with *EcoRV* from type I Gaucher cells infected with the glucocerebrosidase retrovirus at early viral passage. Size markers are λ DNA digested with *HindIII*. The probe employed and methods are described elsewhere (16).

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